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CHANGES IN MEMBRANE FLUIDITY DURING THE MICELLE FORMATION DETERMINE THE EFFICIENCY OF THE SOLUBILIZATION OF MUSCARINIC RECEPTORS

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Abstract. The influence of various concentrations of 15 detergents at different temperatures on the fluidity of membranes was analysed by fluorescence anisotropy of inserted 1,6-diphenyl-1,3,5-hexatriene in rat cortical and *Sf*9 cell membranes. Comparison of the obtained data with the abilities of the detergents to solubilize muscarinic receptor subtypes indicated that not only the fluidity of the micelles formed but also the micelle formation process, the lipid/protein ratio, and presence of sterols have significant influence on the efficiency of the solubilization of mAChR. Digitonin seems to exploit efficiently all these factors by its biphasic modulatory effect on membrane fluidity during the receptor solubilization.

Key words: membrane fluidity, solubilization, detergents, muscarinic receptors, rat cerebral cortex, digitonin, cholesterol.

INTRODUCTION

Detergents are widely used as solubilizing agents for the isolation, characterization, and purification of membrane proteins. They allow detailed study of the properties of membrane-integrated proteins in proteomeric and self-associated states as well as in their interactions with other proteins [1]. For efficient use of detergents it is necessary to know how they interact with integral membrane proteins and lipids and which factors are important for the retaining of active conformation of the proteins. The muscarinic acetylcholine receptors (mAChR), which are members of the family of receptors that are coupled to

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guanine nucleotide binding proteins (G proteins) and whose structure is characterized by the seven membrane-spanning α-helical domains [2, 3], are usually solubilized in digitonin or in a mixture of digitonin and sodium cholate, which have yielded more than 50% of the soluble receptors with retention of ligand binding activity [4, 5]. Sucrose monolaurate, dodecyl-β-D-maltoside, and CHAPSO have also produced high yields in mAChR solubilization, but the stabilities of ligand-binding activities of the preparations of these detergents were lower than when digitonin was used [6–8]. Comparison of physical properties of different detergents has proposed that the fluidity of detergent micelles may play an important role in retaining the active conformation of the solubilized mAChR [9]. However, it was shown that successful solubilization of membrane proteins depends also on the lipid composition of membranes and lipid:detergent and protein:detergent ratios during solubilization [10].

We studied the influence of different concentrations of various detergents and temperatures on the fluidity of membranes, and compared these data with the ability of the detergents to solubilize mAChR. It was found that not only the fluidity of micelles but also the micelle formation process has significant influence on the efficiency of the solubilization of mAChR.

MATERIALS AND METHODS

3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS) and 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO) were from Calbiochem (Darmstadt, Germany); N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulphonic acid) (Hepes), tris(hydroxymethyl)amino-methane (Tris), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1,6-diphenyl-1,3,5-hexatriene (DPH), sodium dodecyl sulphate (SDS), phenylmethylsulphonyl fluoride (PMSF), aprotinin, benzamidine, digitonin, cholic acid, deoxycholic acid, *n*-dodecyl-β-D-maltoside (DBM), and dodecylpoly(ethylene-glycolether)$_n$ (Lubrol PX) were from Sigma Chemical Co. (St. Louis, MO, USA); ethylenediaminetetraacetic acid (EDTA) and inorganic salts were from Merck KGaA (Darmstadt, Germany); Triton X-100, Triton X-114, Tween 20, and Tween 80 were from Ferak (Berlin, Germany); sucrose monolaurate (SM-1200) was kindly donated by Mr. Hajime Machida (Mitsubishi-Kasei Food Co., Japan).

Rat cerebral cortices were homogenized in 20 volumes (wet weight/volume) of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a glass-teflon homogenizer, and centrifuged at $30\,000 \times g$ for 20 min at 4 °C. The membrane pellet was resuspended in 20 volumes (ww/v) of fresh buffer and centrifuged under the same conditions. The final pellet was homogenized in a buffer containing 20 mM K-Hepes, 100 mM NaCl, and 5 mM $MgCl_2$ (pH 7.4 at 21 °C) at a concentration of 25–30 mg wet weight per mL, which corresponded approximately to

1.2–1.5 mg protein per mL as determined by the modified Lowry method [11] with bovine serum albumin used as standard. The aliquotes of homogenate were stored at –80 °C.

The *Sf9* cells expressing different mAChR subtypes were grown as described in [5] by Dr. J. Näsman at the Department of Physiology, Uppsala University (Sweden) and were kindly donated for the present study. The cells were resuspended in a homogenization buffer (4×10^8 cells per 50 mL) containing 20 mM Tris-HCl (pH = 7.4), 5 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 1 μg/mL aprotinin, 0.25 mM benzamidine and homogenized using a Potter-type glass-teflon homogenizer. The homogenate was centrifuged at $27\,000 \times g$ for 40 min at 4 °C and the membrane pellet was washed for two more times by homogenization and centrifugation as described above. The final pellet was resuspended in the 20 mL homogenization buffer and stored at –80 °C.

Fluidity of membranes was measured by the determination of fluorescence polarization of DPH in the membrane suspension [12, 13]. For the insertion of the marker, the obtained suspension of cortical membranes was incubated with 1 μM DPH for 30 min at 37°C. Stock solutions of DPH were prepared in tetrahydrofurane. For the measurements the membranes were diluted 4-fold with the incubation buffer in the presence of different concentrations of the detergents and the fluorescent polarization of DPH was determined at various temperatures with an LS50 spectrofluorometer (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) using excitation and emission wavelengths of 360 and 430 nm, respectively. The slit widths of both the excitation and emission window were kept at 10 nm. The fluorescence anisotropy (r) was measured according to the Perrin equation:

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}},$$

where $I_{\parallel}$ and $I_{\perp}$ are the emission intensities detected by the analyser oriented parallel or perpendicular to the direction of polarization of the excitation light, respectively, and G is the instrumental grating factor.

RESULTS

Fifteen detergents were used. The set was composed to cover a wide range of detergents proposed earlier for solubilization of membrane-bound proteins and it included various non-ionic, ionic, as well as zwitterionic compounds. The abbreviations, chemical names, and properties according to the literature and suppliers are listed in Table 1.

Table 1. Properties of detergents at 25 °C

Detergent		Studied conc., mM	Molecular weight	CMC, mM	Ref.
Abbreviation	Name				
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulphonate	0.05–16	614.9	6–10	[14]
CHAPSO	3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate	0.05–16	630.9	8	[1, 15]
DBM	*n*-Dodecyl-β-D-maltoside	0.02–6	510.6	0.15	[14]
Digitonin	Digitonin	0.01–16	1229.3	0.09	[14]
Genapol X-80	Isotridecylpoly(ethylene-glycolether)$_n$	0.02–5	553 (avg)	0.06–0.15	[15]
Na-cholate	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid sodium salt	0.07–23	430.6	4	[14]
Na-deoxy-cholate	3α,12α-Dihydroxy-5β-cholan-24-oic acid sodium salt	0.24–7	414.6	1.5	[14]
SDS	Sodium dodecyl sulphate	0.10–10	288.4	1.2–10	[14, 15]
SM-1200	Sucrose monolaurate	0.02–6	524.6	0.3–0.7	[1, 15]
Sulfobetaine SB-12	N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulphonate	0.09–30	335.6	1.4–4	[1]
Thesit (Lubrol PX)	Dodecylpoly(ethylene-glycolether)$_n$	0.02–5	583 (avg)	0.1	[1]
Triton X-100	Octylphenolpoly(ethylene-glycolether)$_n$	0.02–5	650 (avg)	0.3	[14]
Triton X-114	Octylphenolpoly(ethylene-glycolether)$_n$	0.02–6	537 (avg)	0.35	[14]
Tween 20	Poly(oxyethylene)$_n$-sorbitane-monolaurate	0.01–2	1228 (avg)	0.06	[14, 15]
Tween 80	Poly(oxyethylene)$_n$-sorbitane-monooleate	0.01–2	1310 (avg)	0.012	[15]

Addition of the detergents to rat cortical membranes caused a decrease in the anisotropy of DPH fluorescence, which was interpreted as an increase in the fluidity of the membranes. The effect of detergents was clearly dependent on incubation temperature. The anisotropy of the polarization DPH as the indicator of the fluidity of membranes decreased with increasing temperature; the dependence was linear between temperatures 4 °C and 37 °C, having no break points in reciprocal coordinates either (data not shown). An increase in temperature also increased the effect of detergents on the fluidity of membranes,

while the shape of concentration-dependent changes did not vary in the case of all detergents studied.

From the 15 different detergents studied only digitonin had a clearly biphasic influence on the membrane fluidity measured by the anisotropy of DPH at all temperatures studied (Fig. 1a). At the detergent concentrations below the critical micelle concentration (CMC) the fluidity of membranes increased significantly. Increase in the detergent concentration above the CMC caused digitonin rigidization of membranes and formation of micelles that were even more rigid than the cortical membranes themselves at the same temperature (Fig. 1a). In comparison with other detergents, digitonin has been reported to be also the best in solubilization of most mAChR subtypes [5, 16].

However, the m2 subtype of mAChR from atrial membranes has been solubilized with sucrose monolaurate and DBM with an even higher yield than with digitonin [6, 7]. In rat cortices these detergents had only a moderate influence on the fluidity of membranes at 4 °C, keeping the value of anisotropy close to that found for natural membranes (Fig. 1b). Increase in temperature led also to a detergent-dependent increase in membrane/micelle fluidity, but the total change did not exceed 0.1 units in fluorescence anisotropy even at the detergent concentration 20-fold above CMC of sucrose monolaurate (Fig. 1b) or dodecyl maltoside (data not shown).

A group of detergents that has been also reported to be able to solubilize active mAChR are zwitterionic CHAPS and CHAPSO [17, 18] and Sulfobetaine SB-12. The solubilization yields with these detergents have remained at considerably lower levels than in the cases of the detergents mentioned above. Below CMC these detergents had no significant influence on the fluidity of cortical membranes, while during the formation of micelles the fluidity increased sharply. When micelles were formed, the tendency to form more rigid micelles at higher detergent concentrations appeared (Fig. 1c).

There are also available other non-ionic detergents, which are widely used for solubilization of membrane proteins, which have not been found suitable for solubilization of integral proteins with several membrane spans. In our study we used Triton X-100, Triton X-114, and Lubrol PX. It has been reported that mAChR in complex with [^{3}H]QNB can be solubilized with reasonable yield with these detergents [16], while without a bound ligand the receptors were inactivated [18]. The influence of these detergents on the fluidity of membranes/micelles was monophasic without clear changes during the micelle formation in the case of all detergents of this group (Fig. 1d or data not shown). These detergents formed micelles with lower anisotropy than the detergents of the previous groups, supporting the idea that the fluidity of the micelles formed has an important role in keeping active conformations of membrane proteins [9].

Ionic detergents such as SDS, sodium cholate, and sodium deoxycholate, but also non-ionic detergents Tween 20, Tween 80, and Genapol X-80 form one more group. All these detergents formed very fluid micelles, reaching the level

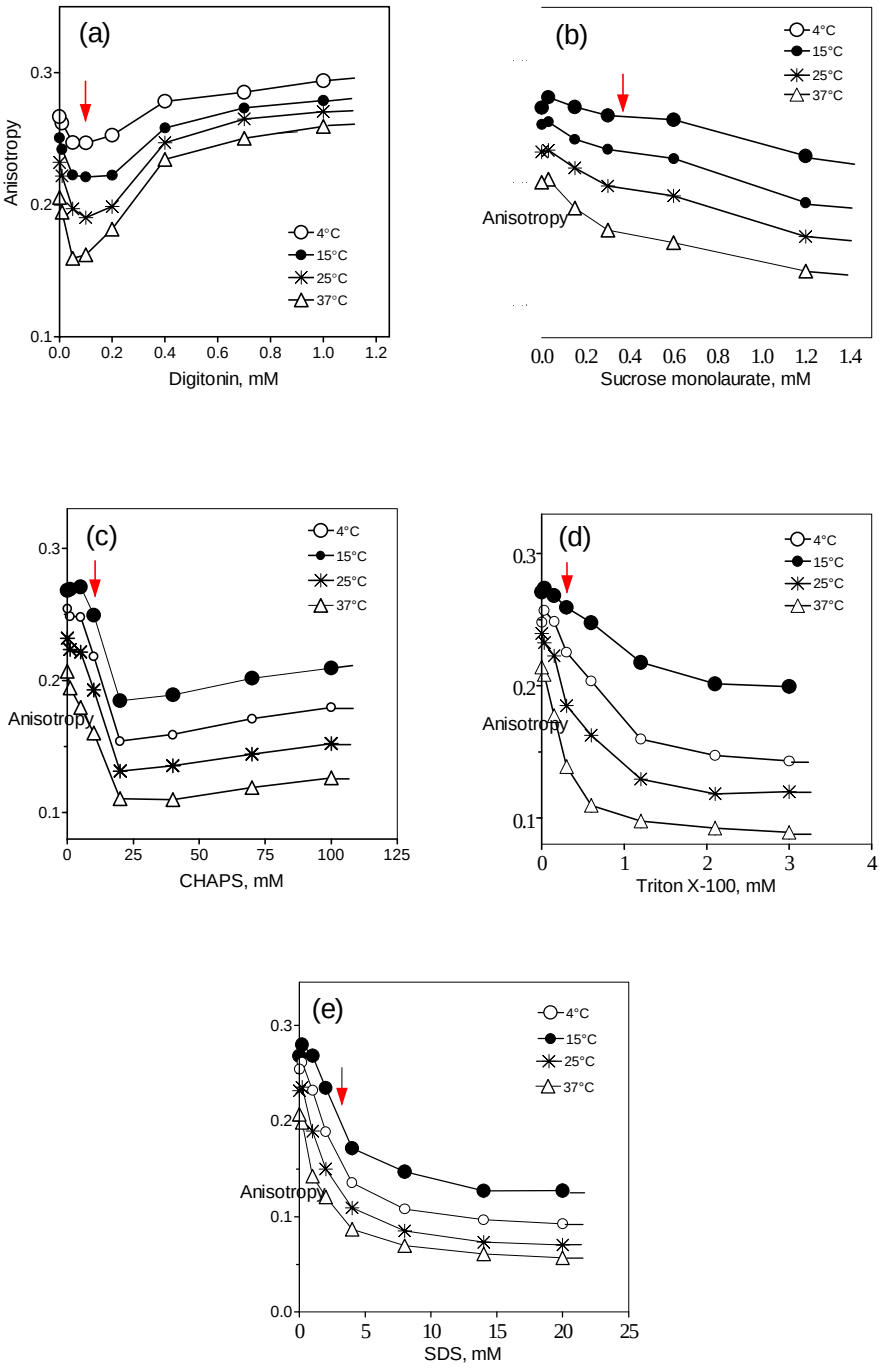

Fig. 1. The effect of detergents on the fluidity of membranes from the rat cerebral cortex. The anisotropy (*r*-value) of the fluorescence polarization of DPH was measured as described in Materials and Methods and is presented as functions of temperature and detergent concentration. The pointers indicate the value of CMC for the detergents.

0.1 of fluorecence anisotropy already at 15°C (Fig. 1e). It has been also shown that all these detergents were effective in solubilization of membrane proteins, but none of them was effective in solubilization of mAChR even in its complexes with a specific radioligand [16, 18].

In our previous work we proposed that the fluidity of detergent micelles plays an important role in muscarinic receptor solubilization, as we found digitonin, the best solubilizer of mAChR, to form highly rigid micelles in comparison with all other detergents studied [9]. In this study we found that digitonin had also biphasic influence on the membrane fluidity. Taking into account digitonin's specific property to form a tight complex with cholesterol [14], it can be proposed that the apparent increase in membrane fluidity is connected with a specific interaction between cholesterol and digitonin. To check this possibility we measured the influence of some detergents on the fluidity of low-cholesterol *Sf9* cell membranes expressing different mAChR subtypes. *Sf9* cell membranes themselves were highly fluid with anisotropy values 0.215 at 4°C to 0.129 at 37°C in comparison with corresponding anisotropy values of 0.267 at 4°C and 0.206 at 37°C for cortical membranes. Addition of digitonin to *Sf9* cell membranes caused a monophasic decrease in the fluidity of membranes/micelles, which reached the value characteristic of digitonin micelles at the same temperature (Figs. 1a, 2a). At the same time, there were no qualitative changes in the influence of other detergents in the case of cell membranes, with only micelles formed at lower detergent concentrations as shown in the case of SDS (Fig. 2b).

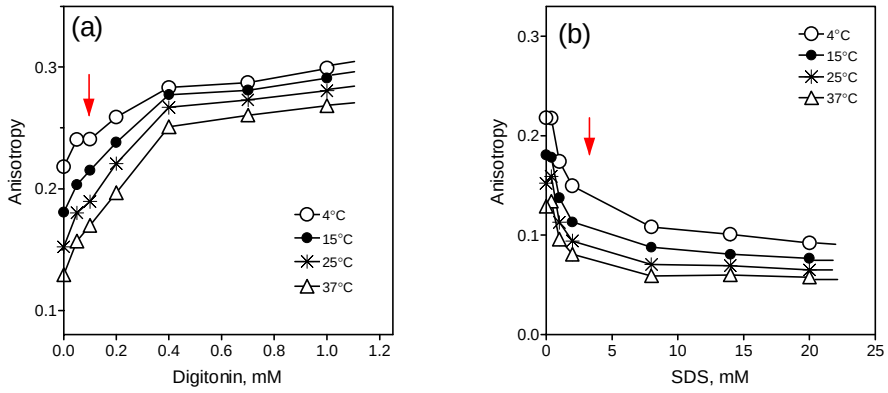

Fig. 2. The effect of digitonin (a) and SDS (b) on the fluidity of *Sf9* cell membranes. The anisotropy (*r*-value) of the fluorescence polarization of the incorporated DPH was measured as described in Materials and Methods. The pointers indicate the value of CMC for the detergents.

DISCUSSION

Cell membranes comprise a lipid bilayer consisting mainly of phospholipids and cholesterol. The functionality of proteins embedded within this environment is essentially affected by the physico-chemical properties of the surrounding membranes. Often the physical state of membranes is characterized by fluidity, which is defined as the relative motional freedom of lipid molecules within the bilayer [19]. The steady state fluorescence polarization techniques using DPH as fluorescence probes have proved a reliable method for the characterization of membrane fluidity in many studies [13, 20]. It is shown that fluorescence anisotropy is proportional to the order of molecular packing and inversely proportional to membrane fluidity, thus directly usable for the characterization of the properties of membranes [19]. In the present study the fluorescence polarization of DPH was used for the characterization of the influence of different detergents on the fluidity of rat cortical membranes during mAChR solubilization.

In general, the addition of detergents to rat cortical membranes caused a decrease in the fluorescence anisotropy indicating an increase in the fluidity of membranes (Fig. 1). It has to be noted that despite quite clear definitions of solubilization in biochemistry [21], there is dynamic equilibrium between membranes with incorporated molecules of detergents and mixed micelles, and therefore we cannot make here a sharp distinction between membranes and micelles. Therefore we discuss here the fluidity of membranes/micelles with an understanding that at concentrations of the detergent above its CMC the equilibrium is shifted toward the formation of micelles.

Comparison of the influence of different detergents on the fluidity of membranes and the fluidities of the micelles formed with the abilities of these detergents to solubilize G protein-coupled receptors allowed us to distinguish five different groups of detergents (Fig. 1a–e). One of the general principles of this grouping was the fluidity of the micelles formed, as more rigid micelles have a tendency to give higher stability to the active conformation of the protein and so higher solubilization yield of active receptors. As all the ionic detergents used formed very fluid micelles, it was difficult to make clear conclusions about the role of the surfactant ionization on the properties of solubilized mAChR. However, as cholate is used as addition to digitonin to increase its solubility during the solubilizations [5] and SDS solubilizes mAChR–[^{3}H]QNB complexes at a very narrow concentration interval [16], it can be proposed that ionization of the detergent by itself does not play a crucial role in the solubilization of mAChR. Substantial solubilization of mAChR has been achieved also using zwitterionic detergents such as CHAPS and CHAPSO [8, 22], but the yields of the active receptors remained usually below the yield obtained with effective non-ionic detergents [18]. Zwitterionic detergents cause a fast increase in the fluidity of membranes during the formation of micelles, while after the formation

of micelles even some rigidization can follow at higher detergent concentrations (Fig. 1c). There were no substantial differences between fluidities of micelles of CHAPS and sucrose monolaurate at the detergent concentrations corresponding to their $10 \times$ CMC, but the mAChR solubilization yield of sucrose monolaurate was 3–5-fold higher [5, 18]. It can be proposed that the fast increase in membrane/micelle fluidity during the micelle formation is a critical property of the zwitterionic detergents, which destabilizes receptors' active conformation and thus causes a decrease in the receptor solubilization yield. This proposal is also supported by the report that CHAPS requires a high lipid/protein ratio for effective solubilization of G protein-coupled receptors [23]. As this ratio is favourable neither in the cortical nor in the *Sf9* cell membranes, CHAPS was quite ineffective in mAChR solubilization from these membranes [5, 16].

The last decade has produced two new detergents, sucrose monolaurate and DBM, which have been reported to be very efficient in the solubilization of G protein-coupled receptors, including mAChR [6, 7]. Here we found that these detergents form quite rigid micelles and the change of the fluidity of the receptors' surrounding is not fast (Fig. 1b). The change of membrane fluidity by addition of these detergents indicated that there is no clear transition from membranes to micelles and solubilization occurs by smooth equilibrium. This makes it possible to keep active conformation of the receptors during the micelle formation and so yields efficient solubilization. This smooth transition would be also the reason why sucrose monolaurate was most effective for the formation of the functional complexes of mAChR with G proteins [18]. However, sucrose monolaurate was very efficient also for solubilization of other proteins from the membranes, causing so several complications in the purification of the receptors [6].

The plant glucoside digitonin differed from all other detergents used in the study in causing biphasic changes in membrane fluidity. At lower concentrations digitonin increased significantly the fluidity of membranes, but during the formation of micelles anisotropy of fluorescence polarization started to increase and finally it formed micelles that were more rigid than the natural membranes (Fig. 1a). Still, digitonin has been the most efficient detergent for the solubilization and purification of G protein-coupled receptors during the last two decades [24]. The unusual behaviour of digitonin during protein solubilization has been found in several studies, while the mechanism of this influence has remained unclear. One possible explanation is connected with its heterogeneous composition [25], but comparison of different digitonin preparations has not revealed any special importance of some components out of the four found in all commercial preparations [26]. As digitonin is a non-ionic detergent, which forms tight, one to one complexes with cholesterol [14], the biphasic influence may be connected with this sterol. Cholesterol increases the fluidity and decreases the order of phospholipids in the gel phase of membranes, but it decreases the fluidity and increases the order of phospholipids in liquid–crystalline state

membranes [27]. It can be proposed that at low concentrations digitonin picks up cholesterol from membranes and so increases their fluidity. At a critical point this causes transition of membranes from gel phase to liquid–crystalline phase, where digitonin can insert into membranes and form rigid micelles, important for keeping active conformation of G protein-coupled receptors [28]. The role of cholesterol on the biphasic influence of digitonin is supported by the finding that in *Sf9* cell membranes, which have a low cholesterol level, the influence of digitonin was monophasic, lacking the fluidity-increase phase (Fig. 2a). The fact that solubilization yields of mAChR from *Sf9* cells were lower than from natural membranes and required a substantial amount of cholate [5] indicates also the essential role of sterols in the solubilization of mAChR, although Furukawa & Haga [29] reported that cholesterol and other sterols are not necessary for the functioning of these receptors in membranes. Additional systematic studies are required to clearly determine the role and mechanisms how cholesterol supports the keeping of active conformation of the G protein-coupled receptors during the solubilization.

To summarize, the nature of changes in the fluidity of membranes during solubilization together with the rigidity of the micelles formed plays an important role in the solubilization of G protein-coupled receptors. However, the mechanism of surfactants, cholesterol, and other membrane components in keeping active conformation of the receptors remains still to be elucidated.

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MUSKARIINSETE RETSEPTORITE SOLUBILISEERIMISE EFEKTIIVSUST MÄÄRAVAD MUUTUSED MEMBRAANIDE VOOLAVUSES MITSELLI MOODUSTUMISEL

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Viieteistkümne detergendi mõju membraanide voolavusele uuriti roti ajukoore ja *Sf9* rakkude membraanides, mõõtes 1,6-difenüül-1,3,5-heksatrieeni fluorestsentsi polarisatsiooni anisotroopia muutusi erinevatel temperatuuridel. Saadud tulemuste võrdlus nende detergentide võimega solubiliseerida muskariinse atsetüülkoliini retseptorite alatüüpe näitas, et lisaks tekkiva mitselli jäikusele mängivad olulist rolli muskariinse retseptori efektiivsel solubiliseerimisel ka selle mitselli moodustumise kiirus, lipiidi ja valgu suhe ning steroolide manulus. Digitoniini eristab teistest detergentidest nende faktorite efektiivne ärakasutamine, mis väljendub kahefaasilises mõjus membraanide voolavusele solubiliseerimisprotsessis.